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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Antiestrogens are the most effective and widely administered therapy for the management of breast cancer. Their efficacy has been attributed to their ability to antagonize the estrogen receptor, and the presence of ER in breast tumor biopsy specimens correlates well with responsiveness to antiestrogen therapy. Still, one in four patients with ER-positive tumors do not respond to antiestrogens, while one in six patients with ER-negative breast tumors undergo objective tumor regression following antiestrogen therapy (Witliff, 1984). These clinical observations suggest that alternative mechanisms of estrogen action may regulate the growth and survival of breast tumors. We have provided evidence that estrogen acts independently of the known estrogen receptors, ERα and ERβ, via the G-protein coupled receptor, GPR30, to regulate the EGFR-to-MAP K signaling axis (Filardo et al, 2000; reviewed in Filardo, 2001; Filardo et al, 2001). Moreover, we have shown that the antiestrogens, tamoxifen and faslodex (ICI 182, 780), also trigger GPR30-dependent regulation of this HB-EGF autocrine loop.

Dysregulation of the EGFR-to-MAP K signaling axis is a common occurrence in breast cancer (Slamon et al, 1989, Sivaraman et al, 1997). The subject of this DOD award is to investigate the relationship between GPR30 expression and MAP K activity in breast tumor biopsy specimens obtained at first diagnosis or following antiestrogen or other adjuvant therapies. The results of these studies may lead to a further refinement in assessing responsiveness to antiestrogen therapy.

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SUMMARY.

During the life of this Career Development Award from the DOD (2000-2004), we have published several manuscripts that support the hypothesis that GPR30 is a previously unappreciated membrane associated estrogen receptor. This funding has directly resulted in our ability to forge alliances in industry (consultant with Procter & Gamble Pharmaceuticals) and academia (collaboration with University of Texas Marine Science Institute) that have led to other award mechanisms, including an American Cancer Research Scholar Award. We will submit an October 1 RO1 award to NIH to further define the role of GPR30 in the activation of the EGFR in breast cancer. Again, it is my belief that DOD was a critical "springboard" for other funding opportunities and collaborations.

The goal of this proposal was to determine the efficacy of GPR30 in assessing antiestrogen responsiveness. This hypothesis seems likely since the known ERs, which belong to the nuclear steroid hormone receptor Superfamily, are the "gold standard" for determining adjuvant therapy for primary breast cancer.

At least two manuscripts will be generated directly from this study. The first describes the distribution of GPR30 in breast and other normal human tissues (attached). The second (and possibly third) manuscript correlates the expression of GPR30 to ER and other histopathological markers. This manuscript is in progress and when completed will identify DOD as a funding source for this work.

To some degree, we were unsatisfied, and frustrated, with our ability to readily acquire large numbers of samples that could be stained GPR30 using peptide antibodies. There are several reasons why this occurred but the most significant resulted from changes in key personnel within Surgery and Pathology that were to provide clinical specimens. Shortly after this award, collaborators, Dr Kirby Bland, Chief of Surgery, and Monique DePaepe, Pathology moved from Rhode Island Hospital. Pathology, in particular, underwent a series of changes in direction and clinical samples were not easily obtained for our purposes.

More recently, Dr Ronald DeLellis moved back to Brown University and as Chair brought with him expert Pathology support personnel that facilitated interactions with basic science and generated funds (COBRE core) that resulted in instrumentation key to this project (tissue arrayer).

GPR30 expression in normal human tissue.

Survey of normal human tissues.

While the work outlined in the original proposal does not specifically state that we would use peptide antibodies to investigate GPR30 expression in tissues other than breast, this information is vital to the assessment of GPR30 as an estrogen-responsive marker. We have previously reported (last year) that we were able to evaluate GPR30 expression in

commercially available normal tissue microarrays (TMAs) (Ambion Inc). After consultation with in house pathology experts (Dr Murray Resnick, Dr Dilip Giri and Tamako Konkin), it is apparent that these tissue preparations generated a background that was not suitable for our conclusions. In the past year, we have refined our staining procedure and have had ready access to fresh tissue arrays that were produced in house and have enhanced the aesthetics of our results (see manuscript). In that, GPR30 is a novel estrogen receptor, and it has a potentially significant impact in human health and disease, we felt that it was essential to be absolutely certain about our immunohistochemical analysis.

Our results are detailed in the attached manuscript *(J of Histochemistry and Cytochemistry)*. In general, our results indicate that GPR30 is not ubiquitously expressed but rather its expression is highest in reproductive tissues (female and male). High levels of GPR30 are also detected in kidney, skin and bone. Hematopoietic tissues (spleen, tonsil, and lymph node) are negative, as are esophagus, thyroid and lung.

Thus, the tissue distribution of GPR30 is consistent with the concept that GPR30 functions as a membrane-associated receptor that promotes estrogen action.

GPR30 expression in human breast tumor biopsies.

Preinvasive breast cancer. We reported last year that GPR30 expression is common in normal mammary epithelia and in preinvasive breast cancer. Again, we relied heavily upon tissue microarrays prodeced by external sources. These arrays were chiefly provided by the National Cancer Institute Cooperative Breast Cancer Tissue Resource (CBCTR) and again our Pathology group demonstrated to us that we were able to generate cleaner results staining specimens that were arranged *in house*. Our results, in progress, do not appear to be vastly different from what we reported last year, but again because GPR30 has potential significance with regards to breast cancer, we have elected to have higher certainty regarding these results using tissues generated *in house*. Since these samples, as well as those obtained from the CBCTR, have complete medical records, they will be included in a manuscript or two describing the relationship of GPR30 to known histopathological markers in breast cancer.

The value of the CBCTR TMAs is that they are provided in a double- blind fashion. Upon receipt of our scoresheet registering GPR30 expression in each of the grids of the tissue microarray, the NCI sends back to us a detailed analysis of each sample. Included with the histopathological analysis of the samples are clinicopathological markers, such as tumor size, node involvement and steroid hormone receptor status. Similarly, we are collating data derived from breast tumor microarrays built *in house*.

In general, our conclusions (from greater than 400 samples of invasive breast cancer) indicates that GPR30 expression occurs independently from the expression of ER and other well described clinicopathological markers for breast cancer.

Last year we reported that after reviewing 188 cases (we are waiting to include data from our in house arrays before publication), no correlation between GPR30 expression, tumor size, node involvement or steroid hormone receptor status was measured (Table I). However, heterogeneity in the intensity of GPR30 expression in these cases was observed and appeared to be independent of clinical parameter. For example, approximately 30% (23/75) of the tumors examined that are less than 2 cm lack GPR30, with the remaining 70% of these samples registering as positives on a scale of +1 to +3. 25% of the node negatives (12/50) and 22% of the node positives (11/49) fail to express GPR30, while the remaining node positive and node negative cases maintain GPR30 expression. A similar trend was observed when comparing GPR30 expression to steroid hormone receptor status. Twenty eight percent of ER+ tumors (27/98) lacked GPR30. By comparison, 27% of ER- tumors (15/55) did not express GPR30. These data strongly indicate that GPR30 expression occurs independently of ER.

Collectively these data are consistent with our hypothesis that GPR30 may serve as an independent marker for assessing antiestrogen responsiveness.

KEY RESEARCH ACCOMPLISHMENTS.

1. As a direct consequence of this funding instrument, immunohistochemical data regarding the expression of GPR30 in normal human tissue and in a significant number of breast cancer biopsy specimens (greater than 200). This data will lead to at least two manuscripts in the upcoming year. Additional manuscripts have been submitted to the Journal of Cell Biology and the Proceedings of National Academy of Sciences regarding the biochemical and biological role of GPR30. Some of the data presented in these manuscripts have been presented at three scientific symposia. Our results concerning the expression of GPR30 in human tissue and our studies concerning the biological role of GPR30 provide the basis for our ongoing collaboration with Procter and Gamble (see Reportable Outcomes below).

2. We continue to use tissue microarray (TMA) slides from the National Cancer Institute Cooperative Breast Cancer Tissue Resource (CBCTR). And very recently (within the past few months) have made use of TMAs that are produced at RIH with a tissue arrayer that was purchase by Pathology by a COBRE funding award. Women and Infant's Hospital in Providence has also very recently within the past few months been able to provide TMAs suitable for immunohistochemical analysis. A single TMA slide contains as many as 200 archival specimens that have been generated from 0.6mm core samples of these tissues. TMAs are provided with histopathological diagnoses that have been provided by board- certified pathologists that work with the National Human Genome Research Institute. Other clinicopathological (tumor size and grade, node involvement, distant metastases, etc) and molecular markers (ie ER status, HER-2/neu expression) are provided with the TMAs in a double-blind fashion.

We have had good success with utilizing our GPR30 peptide antibodies on TMAs and this tissue resource will greatly facilitate our study in scope and uniformity.

3. Employing similar commercially available TMA composed of normal human tissues, we have expanded our survey of GPR30 expression. While this information is not directly outlined in our proposal, it provides information that is crucial to the assessment of GPR30 as a marker of estrogen-responsive tissue. We have assessed GPR30 expression in a wide array of normal tissues, including but not limited to: male and female reproductive tissues, skeletal bone, brain, hematopoietic tissues, tissues from skin, bone, and the digestive and urinary systems. The results of this survey are shown in the attached manuscript

4. We have analyzed 376 breast preinvasive and malignant breast carcinoma specimens that were collected on CBCTR TMAs for GPR30 expression. We have sorted GPR30 expression by tumor size, node involvement, and steroid hormone receptor status (ER/PR) and presented in Table III. This analysis extends our previous observations based on individual archival breast tumor biopsy specimens acquired from the Rhode Island Hospital pathology database (46 cases). Collectively, when these breast tumor samples are combined, we find no obvious correlation between any of the classic clinicopathological markers and GPR30 expression. This information is consistent with our hypothesis that GPR30 may serve as an independent marker for breast cancer. Further evaluation of samples from the CBCTR of patients receiving adjuvant therapies will help us to determine whether GPR30 has prognostic value in determining antiestrogen responsiveness.

REPORTABLE OUTCOMES (life of this award).

1. Publications:

Filardo, EJ, Quinn, JA, KI Bland and Frackelton, AR., Jr (2000) Estrogen-induced Activation of Erk-1 and Erk-2 Requires the G-Protein-coupled Receptor Homologue, GPR30, and Occurs via Transactivation of the EGF Receptor through Release of HB-EGF. Mol Endocrinol **14:** 1649-1660.

Filardo, EJ, Quinn JA, Frackelton AR Jr, Bland KI(2002) Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol. **16:**70-84.

Filardo EJ. (2002) Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol. **80:**231-238.

Quinn, JA, Graeber, CT, Calabresi, P, **Filardo, EJ.** Transmembrane signaling by integrin promotes estrogen-dependent release of proHB-EGF by the G-protein-coupled receptor, GPR30. J Cell Biol (under review)

Graeber, CT, Quinn, JA, Steinhoff, MM, Resnick, M, DeLellis, RA, **Filardo EJ**. Tissue distribution and characterization of GPR30, a heptahelical receptor that promotes the EGF-like effects of estrogen. (manuscript submitted J Histochemistry and Cytochemistry).

Graeber, CT, Quinn, JA, Kim, D, Steinhoff, MM, **Filardo EJ**. Expression of GPR30, a G-protein-coupled receptor that promotes the EGF-like effects of estrogen, in normal mammary epithelia and invasive mammary carcinoma. (manuscript in preparation)

Pang, Y, Dong, J, Quinn, JA, Graeber, CT, **Filardo, EJ**, and Thomas, P. Membrane binding characteristics of GPR30, a novel estrogen receptor. (manuscript in preparation)

Quinn, JA, Graeber, CT, and Filardo, EJ. Physical interaction between estrogen and the heptahelical receptor, GPR30. (manuscript in preparation)

Book chapter.

Filardo EJ, Quinn JA, and Graeber CT. (2002) Evidence supporting a role for GPR30, an orphan member of the G-protein–coupled receptor superfamily, in rapid estrogen signaling. In *Membrane-associated Steroid Hormone Receptors* pub by Kluwer Press Inc and edited by Cheryl S. Watson

2. Presentations.

We were invited to present our work on GPR30 at:

University of Texas Marine Science Institute. "GPR30: a serpentine membrane receptor that promotes the effects of estrogen in human breast cancer tissue". June 25-28, 2003. Port Aransas, Texas.

Arena Pharmaceuticals. "GPR30: an orphan receptor that promotes estrogen-dependent transactivation of the EGFR". August 10, 2003. La Jolla, CA

West Virginia University. "Estrogen, receptor cross-talk and breast cancer: a cell biological role for the orphan G-protein-coupled receptor, GPR30" September 15, 2003. Department of Molecular Biology and Pharmacology. Morgantown, West Virginia.

University of Rhode Island. " Receptors that promote rapid actions of steroid hormones" September 29, 2003. Department of Biology. Kingston, RI.

Tenth Annual T. J. Martell Cancer Consortium, "Identification of a novel estrogen receptor with prognostic utility in human breast cancer cells", October 18-19, 2003, Alton Jones Campus, University of Rhode Island, West Greenwich, RI

COBRE Series Seminar, "GPR30: a heptahelical receptor that promotes the EGF-like effects of estrogen and has possible prognostic utility in breast cancer". January 22, 2004.

3. Research Awards

The DOD has provided us with the momentum to meet and forge collaborations and alliances, in industry and academia, which will enable us to successfully compete for future NIH funding. On October 1, 2004 R01 proposal that will investigate EGFR transactivation by GPR30 in vivo. In this proposal we will assess whether GPR30 signals via GPR30 in vivo and determine its physiological significance. Results gained from this study will allow us to further define the role of GPR30 as a membrane receptor that potentiates estrogen action.

We continue our work on our Research Scholar Award from the American Cancer Society (July 2002- June 2006) entitled "Estrogen Signaling via GPR30". With certainty, our ability to write and receive this ACS award was a direct result of the Career Development Award supported by the DOD. The ACS grant award will enable us to further investigate the mechanism by which GPR30 transactivates the EGFR. It is complementary in nature to the studies funded by the DOD to examine GPR30 expression in human breast cancer biopsy specimens.

4. Invited Consultant on GPR30 action.

I continue to act as a consultant regarding a possible role of GPR30 action in human disease with Procter & Gamble Pharmaceuticals. One direct by-product of our interaction with PGP is that we have refined our immunohistochemical staining procedures using GPR30 peptide antibodies in archival breast biopsy specimens. In addition, PGP will shortly be making available to us transgenic mice that will enable us to further examine the role of this novel estrogen receptor in breast cancer.

CONCLUSIONS.

The known estrogen receptors, ERα and ERβ, are the best prognostic indicators for determining responsiveness to antiestrogen therapy. Still, one in four patients with ER-positive tumors do not respond favorably to anti-estrogens, while one in six patients with ER-negative tumors exhibit objective tumor regression following antiestrogen therapy (Witliff, 1984). These clinical findings, in conjunction with data demonstrating that

antiestrogens trigger rapid signaling events typically not associated with known ERs (Aronica et al, 1994; Lee et al, 2000; Filardo et al, 2000), raises the possibility that antiestrogens may, in part, exert their antitumor effects via non ER-dependent mechanisms.

It has long been suspected that other receptors, distinct from the ER, may participate in estrogen signaling. However, until recently the physical identity of these receptors has remained unknown. Within the past two years, we have provided data demonstrating that the G-protein coupled receptor, GPR30, acts independently of known ERs to transmit intracellular signals that regulate the EGFR-to-MAP K signaling axis (Filardo et al, 2000; reviewed in Filardo, 2002; Filardo et al, 2001). This signaling axis holds particular significance for breast cancer in that it is frequently hyperactivated in breast cancer. Since antiestrogens also act as GPR30 agonists that regulate EGFR-to-MAP K signaling, the studies designed here will enable us to further determine whether there is a link between GPR30 expression, Erk hyperactivation and antiestrogen responsiveness.

From our examination of greater than 400 human breast tumor specimens, we have determined that there is no apparent correlation between ER status and GPR30 expression in human breast tumors (figure 3) and Table III. These data are consistent with our hypothesis that GPR30 may serve as an independent marker for assessing antiestrogen responsiveness.

REFERENCES.

Filardo, EJ, Quinn, JA, Bland KI, and Frackelton, AR, Jr. (2000). Estrogen-induced Activation of Erk-1 and Erk-2 Requires the G-Protein-Coupled Receptor Homologue, GPR30, and Occurs via Transactivation of the EGF Receptor Through Release of HB-EGF. Molec Endocrinol. **14:** 1649-1660.

Filardo, EJ. (2002). Epidermal Growth Factor Receptor (EGFR) Transactivation by Estrogen via the G-Protein_Coupled receptor, GPR30: a Novel Signaling Pathway with Potential Significance for Breast Cancer. J. Steroid Biochem & Molec Biol. **80:** 231-238.

Filardo, EJ, Quinn, JA, Frackelton, AR, Jr. and KI Bland. (2002) Estrogen action via the G-protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the EGFR-to-MAP K signaling axis. Molec Endocrinol. **16(1):** 70-84.

Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst **90:**1371-1388.

Gradishar, WJ and VC Jordan (1998). Endocrine Therapy of Breast Cancer. In Bland, K. I. and Copeland, E. M., eds., The Breast, 2nd ed., Philadelphia, PA: W.B. Saunders and Co.. pg. 1350-1372.

Katzenellenbogen BS, Montano MM, Ekena K, Herman ME, and McInerney EM. (1997). Antiestrogens: Mechanisms of Action and Resistance in Breast Cancer. Breast Cancer Res. Treat. **44:** 23-38.

Sivaraman VS, Wang H-Y, Nuovo GJ, Malbon CC (1997) Hyperexpression of mitogen-activated protein kinase in human breast cancer. J Clin Invest **99:** 1478-1483.

Wittliff JL (1984) Steroid-hormone receptors in breast cancer. Cancer **53:** 630- 643

Appendices.

A. Tables.

Table I. Correlation between GPR30 expression and clinicopathological markers in human breast tumor biopsy specimens.

B. Manuscript.

Graeber, CT[1], Quinn, JA[1], Konkin, T[2], Giri, D[2], Resnick, M [2], DeLellis[2], RA, Steinhoff[3], M and EJ. Filardo[1]. Characterization and tissue distribution of GPR30, a membrane-associated receptor, that promotes the EGF-like effects of estrogen. J Histochemistry and Cytochemistry.

Table I. Correlation between GPR30 expression and clinico-pathological markers in human breast tumor biopsy specimens.

Tissue type	*GPR30 score*[a]				
	0	*+1*	*+2*	*+3*	*total*
Tumor size					
<2 cm	*23*	*26*	*21*	*5*	*75*
2-3 cm	*10*	*26*	*23*	*1*	*60*
3-4 cm	*6*	*10*	*6*	*1*	*23*
>4 cm	*1*	*2*	*0*	*0*	*3*
invasiveness					
node negative	*12*	*22*	*14*	*2*	*50*
node positive	*11*	*22*	*16*	*0*	*49*
distant metastases	*18*	*18*	*12*	*6*	*54*
hormone receptors					
ER+	*27*	*43*	*25*	*3*	*98*
ER-	*15*	*20*	*15*	*5*	*55*
PR+	*23*	*28*	*23*	*4*	*78*
PR-	*21*	*33*	*19*	*4*	*67*

[a] GPR30 expression was scored on a scale ranging from 0 to +3.

[b] ER/PR status, node involvement, and tumor size was provided by the National Cancer Institute

Journal Histochem & Cytochem

Characterization and tissue distribution of GPR30, a membrane-associated receptor, that promotes the EGF-like effects of estrogen.

Graeber, CT[1], Quinn, JA[1], Konkin, T[2], Giri, D[2], Resnick, M [2], DeLellis[2], RA, Steinhoff[3], M and EJ. Filardo[1*]

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ABSTRACT.

We have previously described GPR30 as an orphan member of the G-protein-coupled receptor superfamily that triggers estrogen-dependent autocrine release of HB-EGF from human breast tumor cells. Here we employ antibodies raised against peptides from specific subdomains of the deduced amino acid sequence of human GPR30 to further characterize this receptor and study its tissue distribution. Consonant with its predicted structure and proposed biological function, peptide antibodies detect GPR30 as a 38 kDa membrane-associated type II glycoprotein. Hemagglutinin-tagged recombinant GPR30 protein exhibited a similar expression profile and confirmed peptide antibody specificity. Expression of GPR30, while widely distributed throughout the body, is not ubiquitous and various levels of expression are found in different tissues. Highest levels of endogenous GPR30 expression are detected in male and female reproductive tissues, including: breast, uterus, ovary, prostate and testis. In contrast, GPR30 is present in low or undetectable levels in, tonsil, lymph nodes, thymus, and thyroid. . In addition, reactivity with GPR30 peptide antibodies in positive tissues was competed with immunizing peptide, but not with control peptide. Thus, the tissue distribution of GPR30 is consistent with the concept that GPR30 functions as a membrane-associated receptor that promotes estrogen action.

Key words: estrogen / G-protein / reproductive tissue

INTRODUCTION.

Estrogens exert their effects on a broad spectrum of target tissues. Not only do their actions regulate the growth and development of female and male reproductive organs but their physiological effects are imparted on bone integrity (Termine and Wong,1998), cardiovascular function (Guzzo, 2000) and the central nervous system (Hurn and Macrae, 2000). Specific receptors for estrogen have been identified and their expression profiles in specific tissues has been used as a means to predict estrogen responsiveness ( ). ERα and ERβ are the most well -characterized estrogen receptors. They belong to the steroid hormone receptor (SHR) superfamily, and like other SHRs, function as hormone-inducible transcription factors (Beato and Klug, 2000). While high levels of ERα are measured in female reproductive tissues ( ), more modest levels of this receptor found in other tissue types, including bone, kidney, brain, and vasculature ( ). Other tissues, such as hematopoietic, respiratory and digestive tissues do not appear to express much ERα ( ). In contrast, ERβ is abundantly expressed in some tissues, such as prostate, that fail to produce significant amounts of ERα which is consistent with the notion that these related receptors may provide functional redundancy. Further refinement regarding our ability to determine the role of each receptor in different estrogen responsive tissue types has been facilitated by the use of ER antagonists and the generation of transgenic mice that lack either one, or both, of these receptors. ER activity is to some extent dependent upon ER expression levels and the presence of various cofactors that support ER functionality ( ). Therefore, it is difficult to determine whether ER expression correlates with ER activity. More recently, *in vivo* assessment of ER

activation has been measured by employing mice carrying luciferase reporter transgenes regulated by estrogen response elements (Lemmen et al, 2004).

It has long been suspected that receptors not related to SHRs also promote the action of estrogen ( ). Perhaps the most compelling argument in favor of alternate receptors for estrogen is the observation that estrogen promotes rapid biochemical signals that are inconsistent with the known function of SHRs. Estrogen action has been linked to activation of second messengers (Nakhla et al, 1990; Morley et al, 1992; LeMallay et al, 1997) and the stimulation of protein (Migliaccio et al, 1996) and lipid (Chambliss et al, 2000) kinases (reviewed in Filardo et al, 2002). Both epidermal growth factor receptors (EGFRs) and G-protein- coupled receptors (GPCRs) have been implicated in rapid estrogen action (reviewed in Filardo, 2002 and Levin, 2003). While EGFRs make sensible candidates for this purpose because they regulate kinase cascades, they are not directly tied to second messenger pathways. On the other hand GPCRs provide a likely alternative explanation for rapid estrogen action because they couple to both second messenger and lipid/protein kinase pathways. Data demonstrating that heterotrimeric G proteins are involved in rapid signaling by estrogen (Le Mellay et al, 1999; Raap et al, 2000) further fuels this hypothesis. Evidence has been provided that ER-related proteins associate with the plasma membrane and form complexes with heterotrimeric G proteins (Wyckoff et al, 2001; Razandi et al, 2003), and thus may function as GPCRs. Likewise Shc (Song et al, 2002), Src (Migliaccio et al, 1996) and the p85 regulatory subunit of phosphatidylinositol 3-OH kinase (Simoncini et al, 2000) have been found in association with ER-related proteins. While it is possible that ER may promote rapid estrogen

signaling, the molecular mechanism by which ER couples to these signaling effectors has yet to be determined..

GPCRs often cross-communicate with EGFRs by a process that is referred to as "transactivation" (Prenzel et al, 1999). This mechanism of receptor cross-talk provides a means by which GPCRs may activate second messengers and promote EGF-like effects. We have published that estrogen acts in an ER-independent manner via an orphan member of the GPCR superfamily, named GPR30, to activate adenylyl cyclase (Filardo et al, 2002) and trigger autocrine release of HB-EGF (Filardo et al, 2000) in human breast cancer cells. These estrogen-mediated, GPR30-dependent signaling events have more recently been shown to be operative in keratinocytes (Kanda and Watanabe, 2003a) and peripheral blood monocytes (Kanda and Watanabe, 2003b). In order to further characterize GPR30 and assess its physiological role in estrogen action, here we have employed peptide antibodies derived from various subdomains of GPR30 to study its distribution in normal human tissues.

MATERIALS AND METHODS.

Cells. Human SKBR3 breast carcinoma and HEK-293 transformed embryonal kidney cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a 1:1 mix of phenol red- free Dulbecco's modified Eagle medium: Ham's F12 medium supplemented with 10% fetal bovine serum, and 50 μg/ml gentamicin.

Tissue. Biopsy specimens of normal human tissue were derived from patients who underwent various surgical procedures at Rhode Island Hospital or Women and Infants Hospital, Providence, RI. Tissues were fixed in formalin-alcohol, paraffin-embedded, and subsequently processed with routine techniques. In some instances, biopsy specimens were cryopreserved at time of harvest in liquid nitrogen.

GPR30 peptide antibodies. Synthetic peptides derived from the deduced amino acid sequence of human GPR30 polypeptide were produced by Multiple Peptide Systems (San Diego, CA). N-TER peptide (GTALANGTGELSEHQQ-C) lies within exodomain I and is proximal to the first transmembrane helix (amino acid residues 15-30). EXO II peptide (HERYYDIAVLC) is from the second extracellular domain between transmembrane helices II and III and includes amino acids (120-130). C-TER peptide (C-AVIPDSTEQ-SDVRFSSAV) comprises the 18 carboxyl terminal residues (358-376). Peptides were covalently coupled to Keyhole Limpet Hemocyanin using the bifunctional cross-linker, MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester), Soluble peptide–conjugates were injected intradermally into the flanks of New Zealand White rabbits as previously described in Filardo et al, 2000. Sera from immunized rabbits were screened by ELISA

for reactivity to immobilized peptide and sera demonstrating titers greater than 1: 16,000 were aliquoted and stored at –70C until use.

Immunohistochemistry. Deparaffinization of formalin-fixed tissues was accomplished by heating the slides to 60C for 1 hour followed by three consecutive extractions in Citrisolv (Fisher, ___). Tissues were then washed in ethanol, rehydrated and heated at 95C for 20 minutes in 0.1M sodium citrate, pH 6.0. Endogenous peroxidase activity was quenched in 3% H_2O_2 and nonspecific binding was blocked using bovine serum albumin. Slides were exposed to sera from preimmune or immune rabbits for 2 hours at ambient temperature and then washed three times in Tris-buffered saline containing 0.2% Tween-20 (TBS-T). Tissue-associated rabbit antibodies were detected by using a dextran-coated polymer containing horseradishperoxidase-conjugated goat anti-rabbit IgG (Envision-plus™) and diaminobenezidine as a substrate (Dako Cytomation, Carpinteria, CA). Nuclei were counterstained using Mayer's modified hematoxylin (PolyScientific, Bay Shore, NY).

Cryostat tissue sections were treated to quench endogenous peroxidase activity and block nonspecific binding as described above. Sera were delivered in 5% normal goat serum diluted in PBS (NGS-PBS) for 1 hour. Slides were then washed in PBS and tissue-associated rabbit antibodies were detected using goat anti-rabbit Alexa-488 fluorochrome (Molecular Probes, Eugene, OR) diluted 1:400 in NGS-PBS. Slides were then washed three times in PBS, once in distilled water and coverslips were then mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, CA) to prevent photobleaching. Confocal images were acquired using a Nikon PCM 2000 microscope

(Nikon Inc. Mellvile, NY) using Argon (488 nm) and green Helium-Neon (543 nM) lasers. Serial optic sections were performed with Simple 32, C-imaging computer software (Compix Inc, Cranberry Township, PA). Z –axis serial sections were collected at 0.5 μM with a 60X PlanApo objective and a scan zoom of 1 X. Images were processed and reconstructed in NIH Image shareware.

Recombinant hemagglutinin (HA)-tagged GPR30 fusion protein. The hemagglutinin (HA) epitope YPYDVPDYA was added to the N-terminus of the deduced full-length amino acid sequence of GPR30 polypeptide using standard recombinant DNA procedures. To accomplish this task, a molecular clone containing the entire GPR30 open reading frame, termed GPR-BR (Carmeci et al, 1997), was used as template in a polymerase chain reaction (PCR) employing forward (5' CACCGAATTCAGAGAC-ATGTACCCATACGACGTCCCAGACTACGCGGATGTGACTTCCAAGCC 3'; HA epitope tag *underlined*) and reverse (5' CAAGCTGTCTAGACGGCACTGCTGAA-CCT 3') primers. PCR was performed at a denaturation temperature of 94C for 30s, followed by annealing at 55C for 60s and extension at 72C for 60s for 30 cycles. The PCR amplified product was then cloned by Topoisomerase I-assisted DNA repair into pcDNA3.1(+)neo expression vector (Invitrogen, La Jolla, CA). In this context, the HA-tag lies upstream of the leader sequence, and encodes an HA-GPR30 fusion protein with a calculated apparent molecular mass of 43, 329 Da.

Detergent extracts and immunoprecipitation. Cell monolayers were washed three times in PBS supplemented with 1 mm $CaCl_2$ and 1 mm $MgCl_2$, pH 8.0. and then lysed in RIPA detergent buffer (100 mM Tris, pH 7.6, 150 mM NaCl, 1% deoxycholate, 1%

Triton X-100, 0.1% sodium dodecyl sulfate, 2 mM phenylmethylsulfonylfluoride, 50 mM NaF, 3.5 mM Na_3VO_4 plus a protease inhibitor cocktail (Complete, Roche Biochemicals, Indianapolis, IN). Crude lysates were clarified by centrifugation and protein concentrations were determined by BCA (Pierce, Rockford, IN). Detergent extracts (500 μg) were precleared with ImmunoPure Protein G agarose beads (Pierce, Rockford, IN) in RIPA and then transferred to a fresh tube and incubated with 15-25 μl of immune peptide sera or an equivalent volume of preimmune rabbit sera for 3 hours at 4°C with continuous mixing. ImmunoPure protein G beads (25 μl of packed beads delivered from a 50% slurry) were then added to the lysates and incubated overnight at 4°C to capture immune complexes. Immunopurification of hemagglutinin-tagged protein was accomplished in a similar manner using 2 μg/sample of anti-HA mAB 6E2 (Cell Signaling Technology, Beverly, MA).

SDS- polyacrylamide gel electrophoresis and immunoblotting. Agarose beads were collected by centrifugation, washed in RIPA detergent, and then resuspended in Laemmli sample buffer containing 700 mM β-mercaptoethanol and incubated for 5 minutes at ambient temperature (Bio-Rad, Hercules, CA). Following reduction, the resultant eluent volume was quantitatively recovered by "pin-hole" elution and loaded directly onto 10% SDS-polyacrylamide gels. Proteins were resolved by electrophoresis and electrotransferred onto nitrocellulose membranes (0.45 uM pore size; Schleicher and Schuell, Keene, NH) as described previously (Filardo et al, 2002). Post transfer, nitrocellulose membranes were blocked overnight in TBS containing 0.5% Tween 20 (TBS-T) and 5% nonfat dried milk. Recovery of GPR30 protein was assessed by probing the milk blocked membrane with rabbit polyclonal GPR30 peptide antibodies (Filardo et

al, 2000) diluted 1:500 in TBS-T containing 5% milk for 2 hours at room temperature. mAB 6E2 was used to detect HA epitope tagged proteins at 1:500 in TBS-T/milk overnight at 4C . Immobilized rabbit or mouse antibody-antigen complexes were visualized using horseradish peroxidase-coupled anti-rabbit or anti-mouse antibodies and enhanced chemiluminesence (ECL) (Amersham, Piscataway, NJ).

RESULTS.

GPR30 triggers estrogen-dependent autocrine release of HB-EGF from human breast tumor cell lines (Filardo et al, 2000; reviewed in Filardo, 2002). To study the distribution of this novel membrane receptor for estrogen in normal human tissues, polyclonal antibodies were raised in rabbits against soluble peptides derived from different subdomains of the human GPR30 protein. Of these antibodies, those generated against peptides from the carboxyl terminus (C-TER) and exodomain II (EXO II), between the second and third transmembrane helix, yielded half-maximal serum titers greater than 1:32,000 as assessed by ELISA to immobilized peptide and were selected for further study.

Immunoblot analysis of whole cell lysates prepared from human SKBR3 breast cancer cells demonstrated that rabbit polyclonal antibodies raised against both GPR30 C-TER and EXO II peptides specifically detect a 38 kDa protein (Figure 1). The apparent molecular mass of this species is similar in size to the predicted molecular mass of the mature GPR30 protein (38,915 Da). As shown here, preimmune antibodies do not detect this species. Moreover, the 38 kDa band is more abundant in SKBR3 breast tumor cells that trigger estrogen-induced EGFR transactivation relative to MDA-MB-231 cells that fail to promote this activity (Filardo et al, 2000). Furthermore, the 38 kDa GPR30 species detected by GPR30 C-TER is more abundant in MDA-MB-231 cells that have been transfected with a cDNA encoding human GPR30 protein (Filardo et al, 2000).

To verify the specificity of these GPR30 peptide antibodies, they were tested for their ability to recognize a hemagglutinin (HA)-tagged recombinant GPR30 protein expressed in human HEK-293 embryonal kidney cells (figure 2). Anti- HA mAB 6E2

was added to detergent extracts prepared from either mock- or HA-GPR30-transfected HEK-293 cells, immune complexes were collected using protein G agarose beads and associated proteins were blotted with GPR30 peptide antibodies. A single molecular mass species with an apparent molecular weight of 41 kDA was detected in HA-GPR30 transfected cells and absent in mock-transfected cells (figure 2A). The relative increase in molecular mass over that of the endogenous GPR30 protein (38 kDA) detected in whole cell lysates (figure 1) is consistent with mass added by the HA epitope and the retention of leader sequences at the N-terminus of GPR30. Similarly, a 41 kDa band was also detected in experiments in which GPR30 peptide antibodies were used for immuno-precipitation and anti-HA antibodies were employed for blotting (figure 2B). Similar results were obtained when either C-TER or EXO II peptide antibodies were used.

GPR30 peptide antibodies were tested for their ability to stain archival paraffin-embedded surgical specimens obtained during reductive mammoplasties. As shown in figure 3B, GPR30 C-TER peptide antibodies stained both ductal epithelium and stromal cells. Neither cell type was stained in an adjacent serial section reacted with preimmune sera (Figure 3A). Within the ductal epithelia, the GPR30 C-TER peptide antibodies were found concentrated at the plasma membrane (Figure 3A), an observation consistent with its predicted subcellular location as a heptahelical receptor. This pattern of staining was observed in surgery specimens acquired from eight different individuals undergoing reductive mammoplasty. Analysis of mammary epithelium obtained from nursing and virgin adult mice, indicates that its expression does not change during lactation (data not shown). Similar reactivities were observed in mammary tissue stained with N-TER or EXO II peptide antibodies (data not shown).

To further evaluate the specificity of the GPR30 C-TER antibody reactivity in mammary tissue, a peptide competition experiment was performed (Figure 3). Immunizing peptide, or control peptide were preincubated for several hours with GPR30 C-TER peptide antibodies prior to delivery to archival normal breast biopsy specimens. The antibody-peptide mixture was then applied to tissue and tissue-associated rabbit immunoglobulin was measured by immunohistochemistry. As shown in Figure 3C, immunizing C-TER peptide (1 μg) significantly reduced the staining of human mammary tissue, whereas 10 μg of control peptide (N-TER), derived from the amino terminus of GPR30, had no effect on GPR30 C-TER peptide antibody staining (Figure 3D). These results suggest that the reactivity of GPR30 C-TER peptide antibody for human breast tissue is specific for the immunizing GPR30 peptide.

To confirm the subcellular staining pattern generated by GPR30 C-TER peptide antibodies in archival specimens, frozen normal breast specimens were reacted with GPR30 C-TER peptide antibodies or preimmune rabbit antibodies, and detected by immunofluorescence. In this analysis, tissue-associated rabbit antibodies were visualized using goat anti-rabbit antibodies conjugated with the fluorochrome, Alexa- 488 nm (green) and nuclei were counterstained with DAPI (red). Fluorescent images were captured by confocal microscopy. As evidenced by the green stain outlining the membrane of both ductal epithelium, GPR30 C-TER peptide antibodies marked GPR30 as a membrane-associated antigen (figures 4A). GPR30 was not detected in the nuclear compartment (figure 4A). No reactivity was observed in control specimens that were incubated with preimmune rabbit antibodies (figure 4B). These data are consistent with the predicted structure and function of GPR30 as a membrane-associated receptor.

A prior study using Northern hybridization analysis indicated that GPR30 is expressed in a variety of tissues (Carmeci et al, 1997). To further study the tissue distribution of GPR30, expression of GPR30 in various human tissues was evaluated by immunohistochemistry using peptide antibodies. As evidenced in figures 5 and 6, and summarized in Table I, expression of GPR30 was not ubiquitous and various levels of GPR30 expression were detected in different tissues. Abundant levels of GPR30 were found in human reproductive tissues (figure 5). In addition to breast, high levels of GPR30 expression were detected in other female reproductive tissues with representative examples of uterus and ovary shown in figures 5A and 5B, respectively. As demonstrated by Northern hybridization (Carmeci et al, 1997), high levels of GPR30 were also observed in placenta (data not shown). More modest levels of GPR30 were observed in cervix (data not shown). GPR30 expression was not limited to female reproductive structures. Male reproductive tissues also express abundant levels of GPR30. A survey of prostate (figure 5C) and testis (figure 5D) indicated that GPR30 is expressed well in these tissues and in seminal vesicles (data not shown).

In contrast, hematopoietic tissues generally expressed low or undetectable levels of GPR30 (figure 6). Tonsil and lymph node (figures 6A and B, respectively) were devoid of GPR30 whereas a slight amount of GPR30 expression was detected in spleen (figure 6C). Thymus was also negative (data not shown). Similarly, infiltrating mononuclear cells in breast tumor samples did not express GPR30 protein (data not shown). It is possible that the GPR30 reactivity expressed in spleen is associated with histiocytes which is consistent with the fact that peripheral blood monocytes express GPR30 (Kanda and Watanabe, 2003). Specimens of lung (figure 6D), esophagus (figure

6E) and thyroid (figure 6F) uniformly expressed little detectable GPR30 protein. GPR30 expression was confined to specific cellular compartments within skin, kidney and pancreas. The upper layers of the dermis were strongly positive for GPR30 (figure 6G) while the deep fascia was negative. In a similar manner, uriniferous tubules of the kidney express elevated levels of GPR30 while cells within the kidney cortex were negative (figure 6H). As demonstrated in this figure, the squamous epithelial cells that comprise Bowman's capsule express little detectable GPR30 and modest amounts of GPR30 are observed within the glomeruli. Acinar cells of the pancreas appear to make little GPR30 protein (figure 6I), however, other portions of this tissue were positive.

As summarized in table I, these observations suggest that GPR30 is not expressed ubiquitously and that its expression is not uniform throughout tissues of the body. Abundant expression is found at highest levels within reproductive tissues, while hematopoietic tissues were generally negative. These data indicate that GPR30 is expressed in a tissue- specific manner and are consistent with a physiological role as a membrane receptor that promotes estrogen action.

DISCUSSION.

Receptors other than ERα and ERβ have been hypothesized to participate in estrogen action. This theory has evolved from the fact that estrogen triggers second messenger cascades and growth factor signaling pathways; rapid signaling events which are inconsistent with the known structure and function of ERs as ligand-inducible transcription factors. We have shown that estrogen acts via GPR30, an orphan member of the G-protein-receptor superfamily, to stimulate adenylyl cyclase activity (Filardo et al, 2002) and promotes autocrine release of proHB-EGF (Filardo et al, 2000). To further study the biological role of GPR30 and its possible function as an alternate estrogen receptor, its tissue distribution was surveyed. Its expression is abundant in normal mammary tissue, and as predicted from its structure and function, it is predominantly located in the plasma membrane (figures 3 and 4). GPR30 is present in both ductal epithelium and in stromal cells in the breast (figure 3). A similar distribution between stromal and mammary epithelial cells is observed for ERα, the most well-studied estrogen receptor, and it is well appreciated from transplantation studies between wild-type and ERα-deficient mice that both stromal and ductal cellular compartments are estrogen responsive ( ). GPR30 expression is equally well represented in other female reproductive tissues including ovary and uterus (figure 5A, B) and placenta (data not shown). In addition to its presence in female reproductive tissues, GPR30 is also expressed at high levels in male reproductive structures (figure 5 C, D). Male tissues express ER proteins and this observation have been associated a negative regulatory effect of estrogen upon the developing male reproductive tract ( ) and the partial efficacy of antiestrogens on cancers that arise from the prostate ( ).

In that EGF and estrogen are potent mitogens in both uterus and breast tissue, GPR30 provides a possible mechanism for mitogenic coupling in these tissues. In fact, neutralizing antibodies to EGF inhibit estrogen-dependent proliferation in the uterus (Nelson et al, 1991). Further testimony indicating that these mitogens may act coordinately is provided by the observation that exogenous EGF promotes extracellular-regulated kinase-1/-2 dependent phosphorylation of serine 118 within the activation function-2 (ATF-2) subdomain of the ER, and thereby enhancing its gene activation function (Arnold et al, 1995; Kato et al, 1995). The fact that GPR30 transactivates the EGFR by triggering the release of membrane-associated proHB-EGF (reviewed in Filardo, 2002), suggests that GPR30 may enhance ER functionality in *trans* by an estrogen-induced autocrine loop mechanism. Likewise, estrogen promotes EGFR activity in the uterus and intrauterine administration of cholera toxin results in uterine hyperplasia ( ), suggesting still yet another mechanism by which GPR30 may promote mitogenic coupling. The molecular mechanism by which estrogen imparts an inhibitory effect on male reproductive tissues remains unclear. In this regard it is interesting to note that GPR30 promotes apoptotic signaling suggesting a possible mechanism by which estrogen may inhibit the development of male reproductive tissue (Ahola et al, 2002).

Not all tissues express GPR30. For example, hematopoietic tissues were generally devoid of GPR30 (figures 6A, B, and C). In general, peripheral blood cells are also considered to be low for expression of ER-related proteins yet various labs have measured signaling events in peripheral blood cells exposed to estrogen ( ). Other reports have indicated that peripheral blood mononuclear cells support GPR30-dependent action (Kanda and Watanabe, 2003) and at least one group has shown that GPR30 is expressed

in peripheral blood cells (Kvingedal and Smeland, 1997). Although some reactivity was measured in spleen (figure 6C), it is not clear from this study whether this is the result of Fc-receptor sequestration of peptide antibodies by histiocytes from this tissue. Specimens from the lung, esophagus and thyroid (figures 6D, E, F), respectively, were also negative for GPR30. These tissues do not produce much ER and are not been described as estrogen responsive.

Transgenic mice carrying an ERE-regulated luciferase have been used to measure estrogen receptor activation in vivo (Lemmen et al, 2004). In these ER reporter mice, kidney exhibits the highest degree of luciferase activity. Results from studies in which glomerular hypertrophy was prevented in ERα knockout mice subjected to experimental diabetes further implicates a physiological role for ERα in the kidney (Lovegrove et al, 2004). We measured elevated levels of GPR30 protein localized to uriniferous tubules (figure 6H). This result is in concert with a prior study that demonstrated by Northern hybridization analyses that low levels of GPR30 transcript were present in mRNA extracted from kidney (Carmeci et al, 1997).

Of the other tissues that were surveyed, elevated levels of GPR30 are also found in the upper dermal layers of skin (figure 6G), including strong reactivity in keratinocytes and fibroblasts. Consistent with this observation, a prior study has shown that cultured keratinocytes exhibit GPR30 activity (Kanda and Watanabe, 2003). Sex steroid hormones, including estrogen contribute to skin elasticity and ERs have been measured within various subcellular components of the skin. Whereas ERα is expressed at low levels throughout the dermis, ERβ is highly expressed in the epidermis, sebaceous and eccrine sweat glands (Pelletier and ren, 2004). Collectively, these data further

demonstrate that GPR30 like the ERs, while widespread in their distribution, are not uniformly expressed throughout various tissues of the body.

In the present study, we have determined that GPR30 is widely distributed throughout the body and that its expression varies greatly between different tissues. In general GPR30 is expressed largely in tissues which express ERa or ERb and are considered to be estrogen responsive. Since GPR30 activates rapid intracellular signaling events that augment ER functionality, the tissue distribution of GPR30 is consistent with the concept that GPR30 is a novel heptahelical receptor for estrogen.

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LITERATURE CITED.

Aronica, SM, Kraus, WL, and Katzenellenbogen, BS (1994) Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription. Proc Natl Acad Sci USA. 91: 8517-8521.

Carmeci, C, Thompson, DA, Ring, HZ, Francke, U. and Weigel, RJ (1997) Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer. Genomics 45: 607-617.

Carpenter, G (1999) Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. J Cell Biol 146: 697-702

Cunha, GR, Wiesen, JF, Werb, Z, Young , P, Hom, YK, Cooke, PS and Lubahn, DB (2000) Paracrine mechanisms of mouse mammary ductal growth. Adv Exp Med Biol. 480:93-97.

Faraldo, MM, Deugnier, MA, Thiery, JP and Glukhova, MA (2001) Growth defects induced by perturbation of β1-integrin function in mammary gland epithelium result from lack of MAP K activation via the Shc and AKT pathways. EMBO reports 2: 431-437.

Filardo, EJ, Quinn, JA, Bland, KI and Frackelton Jr, AR (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G-protein coupled receptor homolog, GPR30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. Molec. Endo. 14: 1649-1660.

Filardo, EJ (2002) Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J. Steroid Biochem & Molec Biol 80: 231-238.

Filardo, EJ, Quinn, JA, Frackelton Jr, AR and Bland. KI (2002) Estrogen action via the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis. Mol Endocrinol. 16:70-84.

Imagawa, W, Pedchenko, VK, Helber, J, and Zhang, H (2002) Hormone/growth factor interactions mediating epithelial/stromal communication in mammary gland development and carcinogenesis. J Steroid Biochem Mol Biol. 80:213-230.

Kanda N, Watanabe S. (2003a). 17beta-estradiol inhibits oxidative stress-induced apoptosis in keratinocytes by promoting Bcl-2 expression. J Invest Dermatol. **121:**1500-1509.

Kanda, N and Watanabe, S (2003b) 17β-estradiol enhances the production of nerve growth factor in THP-1-derived macrophages or peripheral blood monocyte-derived macrophages. J Invest Dermatol **121:**771-780.

Kvingedal AM and Smeland EB. (1997). A novel putative G-protein-coupled receptor expressed in lung, heart and lymphoid tissue. FEBS Lett. **407:** 59-62.

Lemmen, JG, Arends RJ, Van Boxtel AL, Van Der Saag PT, and Van Der Burg B (2004) Tissue- and time-dependent estrogen receptor activation in estrogen reporter mice. J Mol Endocrinol. **32:**689-701.

Levin ER. (2003) Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol. 17:309-317

Lovegrove, AS, Sun, J, Gould KA, Lubahn, DB, Korach, KS and Lane, PH. (2004). Estrogen receptor alpha mediated events promote sex-specific diabetic glomerular hypertrophy. Am J Physiol Renal Physiol (epub)

Maruyama, T, Miyamoto, Y, Nakamura, T, Tamai, Y, Okada, H, Sugiyama, E, Nakamura, T, Itadani , H, and Tanaka, K (2002) Identification of membrane-type receptor for bile acids (M-BAR). Biochem Biophys Res Commun. 298:714-719.

Migliaccio, A, DiDomenico, M, Castoria, G, deFalco, A, Bontempo, P, Nola, E, and Auricchio. F (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J. 15:1292-1300.

Morley, P, Whitfield, JF, Vanderhyden, BC, Tsang, BK and Schwartz, JL (1992) A new, non-genomic estrogen action: the rapid release of intracellular calcium. Endocrinology. **131:**1305-1312.

Pelletier G, Ren L. (2004). Localization of sex steroid receptors in human skin. Histol Histopathol. **19:**629-636.

Prenzel, N, Zwick, E, Daub, H, Leserer, M, Abraham, R, Wallasch C, and Ullrich, A (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of HB-EGF. Nature 402: 884- 888.

Prenzel, N, Fischer, OM, Streit, S, Hart, S, and Ullrich,A (2001) The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. Endocr Relat Cancer **8:**11-31

Raap, DK, DonCarlos, L, Garcia, F, Muma, NA, Wolf, WA, Battaglia, G, and Van de Kar, LD (2000) Estrogen desensitizes 5-HT(1A) receptors and reduces levels of G(z), G(i1) and G(i3) proteins in the hypothalamus. Neuropharmacology. 39:1823-1832.

Razandi, M, Pedram, A, Park, ST, and Levin, ER (2003) Proximal events in signaling by plasma membrane estrogen receptors. J Biol Chem 278:2701-2712.

Schedin, P, Strange, R, Mitrenga, T, Wolfe, P, and Kaeck, M (2000) Fibronectin fragments induce MMP activity in mouse mammary epithelial cells: evidence for a role in mammary tissue remodeling. J Cell Sci 113:795-806.

Simoncini, T, Hafezi-Moghadam, A, Brazil, DP, Ley, K, Chin,WW, and Liao, JK (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature. 407:538-541.

Song, RX, McPherson, RA, Adam, L, Bao. Y, Shupnik, M, Kumar, R, and Santen, RJ (2002) Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. Mol Endocrinol. 16:116-127.

Sternlicht, MD, Lochter, A, Sympson, CJ, Huey, B, Rougier, JP, Gray, JW, Pinkel, D, Bissell, MJ and Werb,Z (1999) The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. Cell. 98:137-146.

Vladusic, EA, Hornby, AE, Guerra-Vladusic, FK, Lakins, J, and Lupu, R (2000) Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. Oncol Rep. 7:157-167.

Wiseman, BS, Sternlicht, MD, Lund, LR, Alexander, CM, Mott, J, Bissell, MJ, Soloway, P, Itohara, S, and Werb, Z (2003) Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. J Cell Biol. 162:1123-1133.

Wyckoff, MH, Chambliss, KL, Mineo, C, Yuhanna, IS, Mendelsohn, ME, Mumby, SM, and Shaul, PW (2001) Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Galpha(i). J Biol Chem. 276:27071-27076.

Zhu, Y., Bond, J and Thomas, P (2003) Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc Natl Acad Sci USA. 100:2237-2242.

FIGURE LEGENDS.

Figure 1. Peptide antibodies detect an endogenous 38 kDa membrane protein in human breast cancer cell lines.

(A) Whole cell lysates from human SKBR3 breast cancer cells were blotted with antibodies purified from rabbits prior to immunization or following immunization with peptides derived from exodomain II (Exo II) or the C-terminus (C-TER) of the deduced human GPR30 amino acid sequence. Molecular mass standards are indicated at left.

Figure 2. Peptide antibodies recognize hemagglutinin-tagged receombinant GPR30 protein.

Total cellular protein was extracted from human HEK-293 kidney cells that were transfected with hemagglutinin (HA)-tagged GPR30 or vector. One milligram of total cellular protein was then immunoprecipitated with C-TER GPR30 peptide antibodies and then blotted with HA-mAB, or conversely was immunoprecipitated with HA-mAB and then probed with C-ter GPR30 peptide antibodies. Immunoprecipitates were incubated in the presence (+) or absence (-) of N-glycosidase F prior to gel electrophoresis.

Figure 3. Inhibition of GPR30 peptide antibody reactivity for human breast tissue with immunizing peptide, but not control peptide.

Normal human mammary tissue specimens were obtained from patients undergoing reductive mammoplasty. Adjacent serial sections from formalin-fixed, paraffin-embedded tissue were exposed to serum collected from preimmune (A) or GPR30 C-ter peptide (B) rabbits. Tissue-associated antibodies, *brown*, were visualized by an immunohistochemical reaction employing a dextran polymer containing anti-rabbit IgG coupled to horseradishperoxidase and diaminobenizidine (DAB) as substrate (Envision-

plus™). Nuclei were identified by counterstaining with hematoxylin, *blue*. GPR30 C-TER peptide antibodies were preincubated with 1 μg of immunizing peptide (C) or control peptide (D) prior to exposure to adjacent serial sections of formalin-fixed normal human mammary tissue. Sections are shown at 200-fold magnification. Bars = 50 μm.

Figure 4. Subcellular localization of GPR30 in frozen breast biopsy specimens. Cryostat sections of normal breast surgery biopsy specimens obtained during reductive mammoplasty were reacted with (A) preimmune or (B) GPR30 C-TER antibodies and visualized using anti- rabbit antibodies conjugated with the fluorochrome Alexa-488, *green*. Nuclei were counterstained with DAPI and assigned a psuedo-color, *red*. Fluorescent images were captured by confocal microscopy and are shown at 600-fold magnification. Bars = 50 μm.

Figure 5. Expression of GPR30 in male and female reproductive tissues. Formalin- fixed normal human tissue from (A) endometrium, (B) ovary, (C) prostate, and (D) testis were immunostained with GPR30 C-TER antibodies, *brown*. Nuclei are counterstained in *blue*. All images are shown at 200-fold magnification except where otherwise noted. Bars = 50 μm.

Figure 6. Expression of GPR30 in various normal human tissues. Formalin- fixed normal human tissue from (A) tonsil, (B) lymph node, (C) spleen, (D) lung, (E) esophagus, (F) thyroid, (G) skin, (H) kidney were immunostained with GPR30

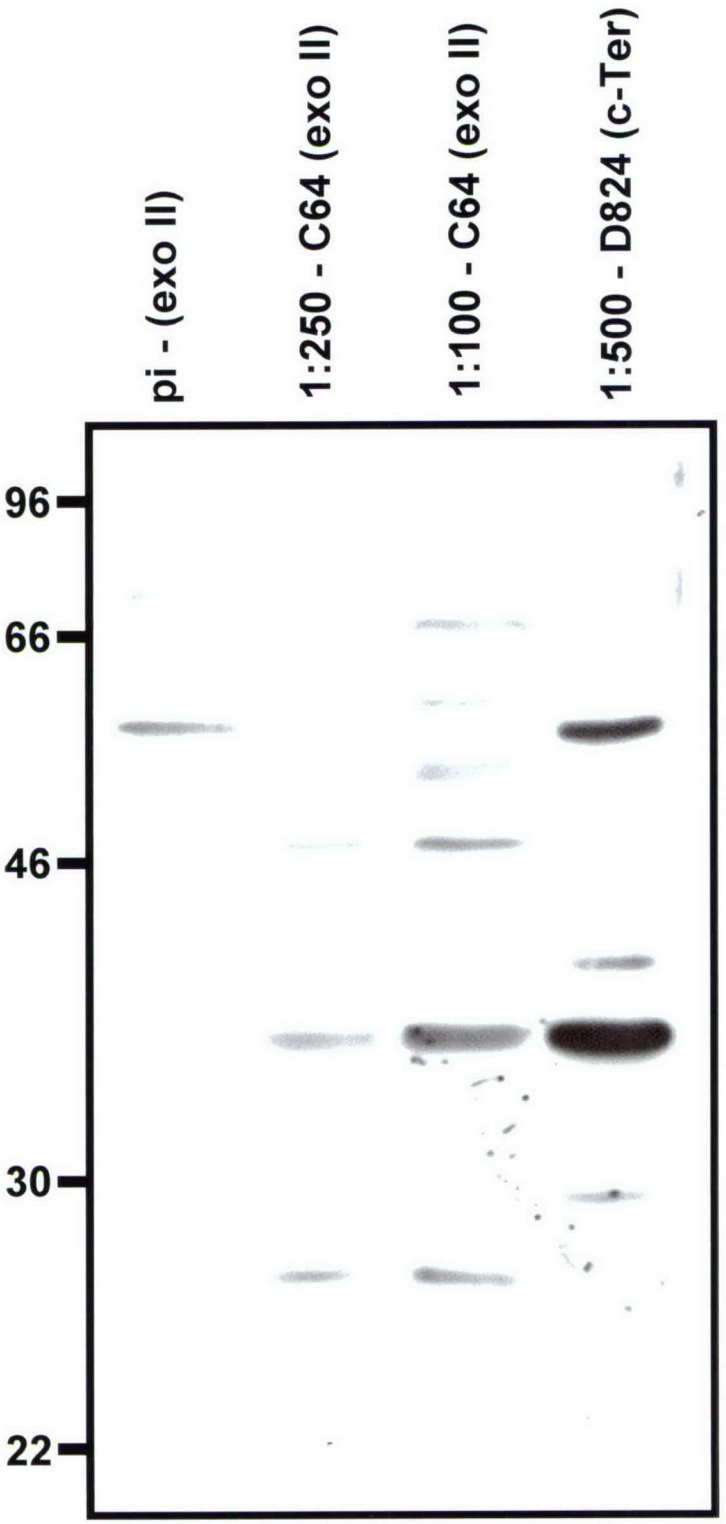
C-TER antibodies, *brown.* Nuclei are counterstained in *blue*. All images are shown at 200-fold magnification. Bars = 50 μm.

Table I. Expression of GPR30 in human tissues.

Data tabulated from normal tissue surgery specimens. Multiple examples of each tissue have been evaluated by at least two independent observers and the intensity of GPR30 expression graded on a scale of 0 to +3.

Table I. GPR30 expression in normal human tissues

Tissue Type	GPR30 expression index
Reproductive tissues	
Breast	*+2*
Endometrium	*+2*
Ovary	*+2*
Placenta	*+2*
Prostate	*+2*
Seminal vesicle	*+2*
Testis	*+3*
Hematopoeitic tissues	
Lymph node	*0*
Spleen	*0*
Thymus	*0*
Tonsil	*0*
Other tissues	
Kidney cortex	*+2*
Skin	*+2*
Appendix	*0*
Cerebrum	*0*
Esophagus	*0*
Heart	*0*
Lung	*0*
Skeletal muscle	*0*
Small intestine	*0*
Thyroid	*0*



SKBR3

Figure 1

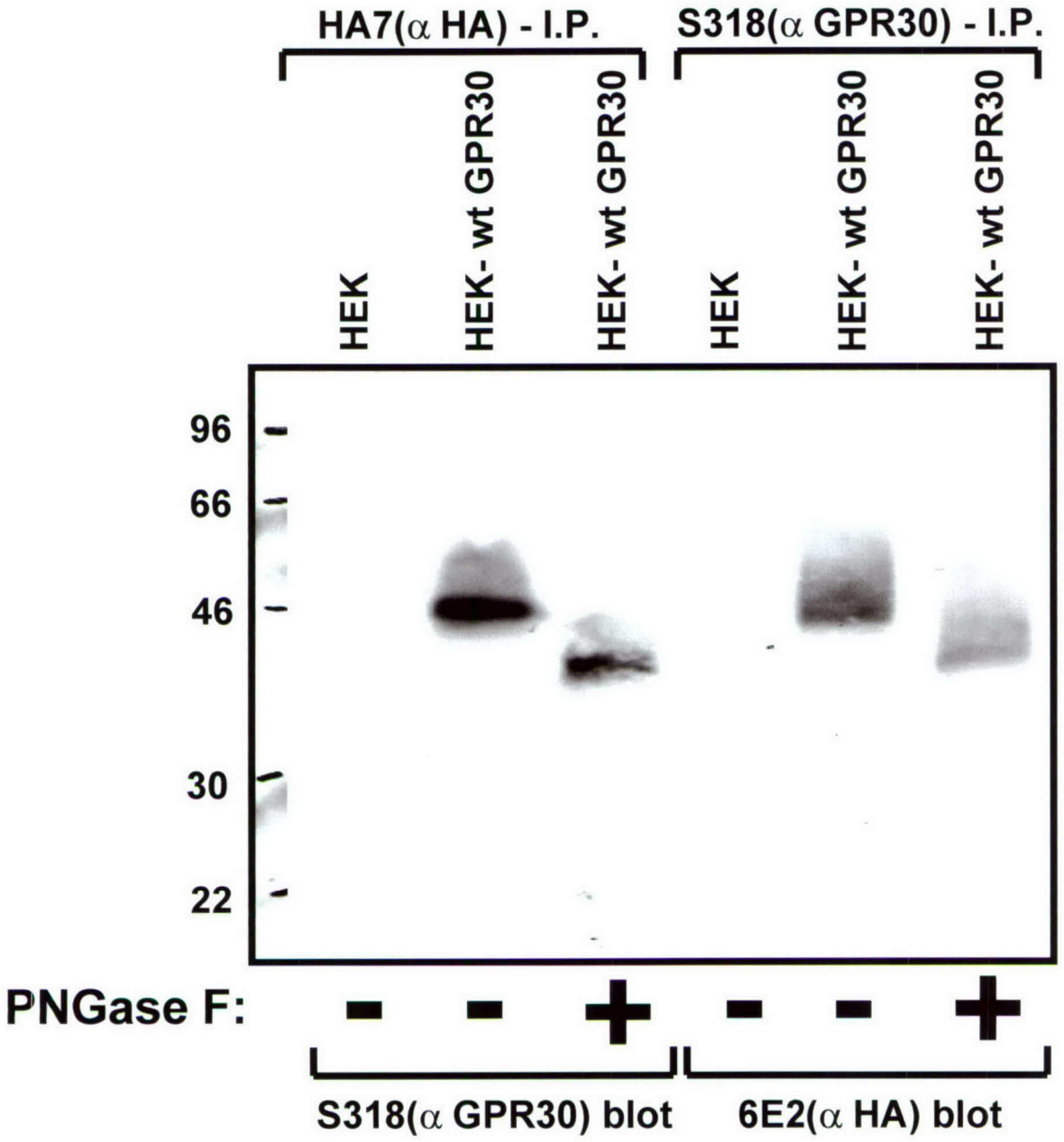

HA7(α HA) - I.P.
S318(α GPR30) - I.P.
HEK
HEK- wt GPR30
HEK- wt GPR30
HEK
HEK- wt GPR30
HEK- wt GPR30
96
66
46
30
22
PNGase F:
- - + - - +
S318(α GPR30) blot
6E2(α HA) blot

Figure 2

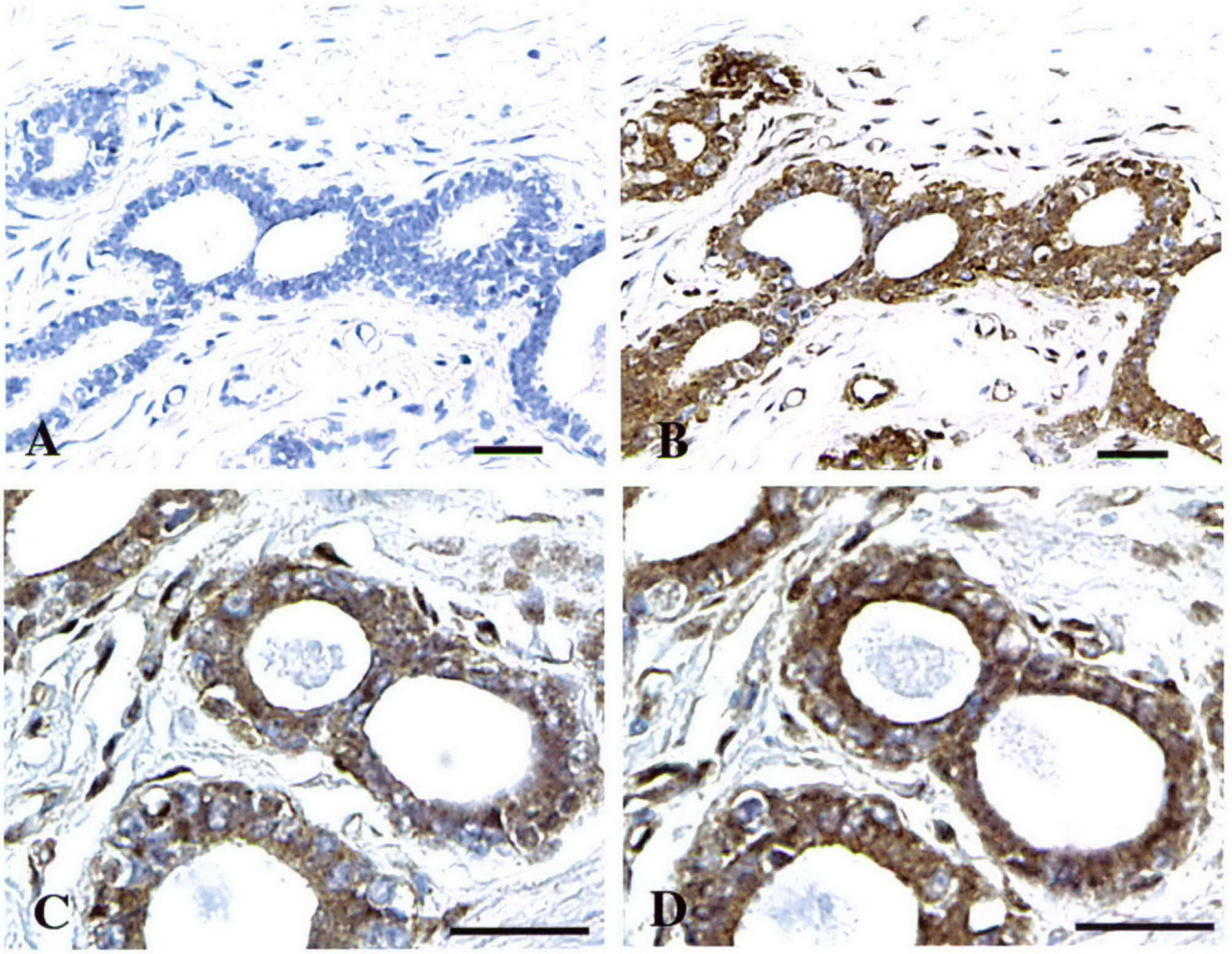


Figure 3

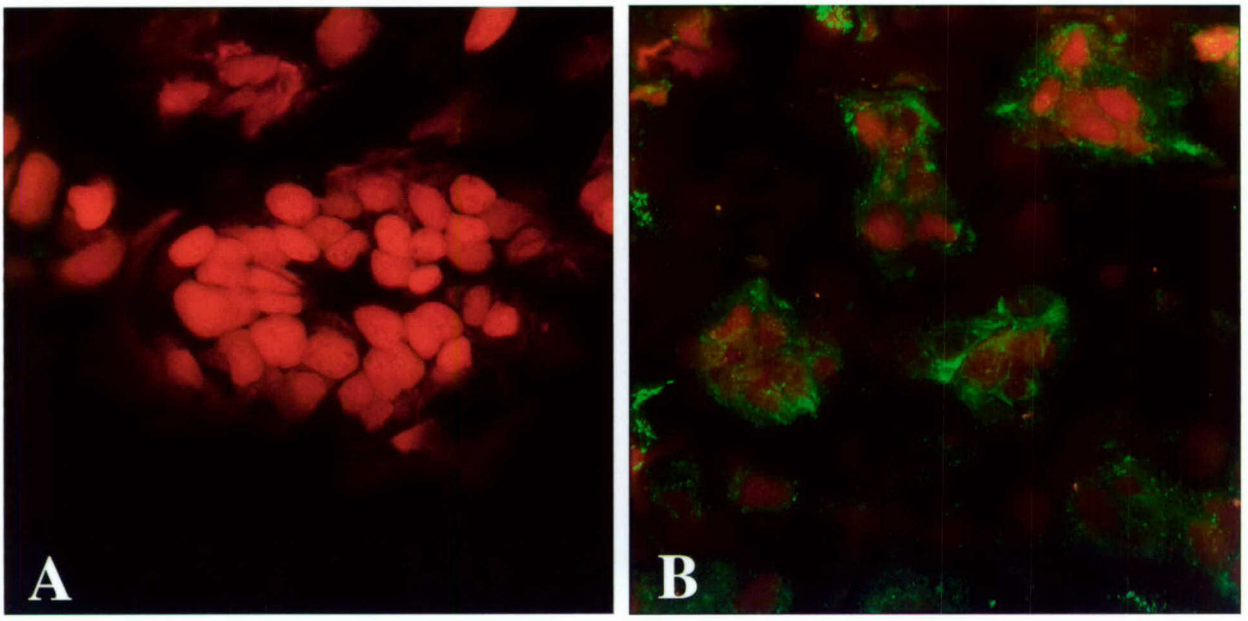


Figure 4

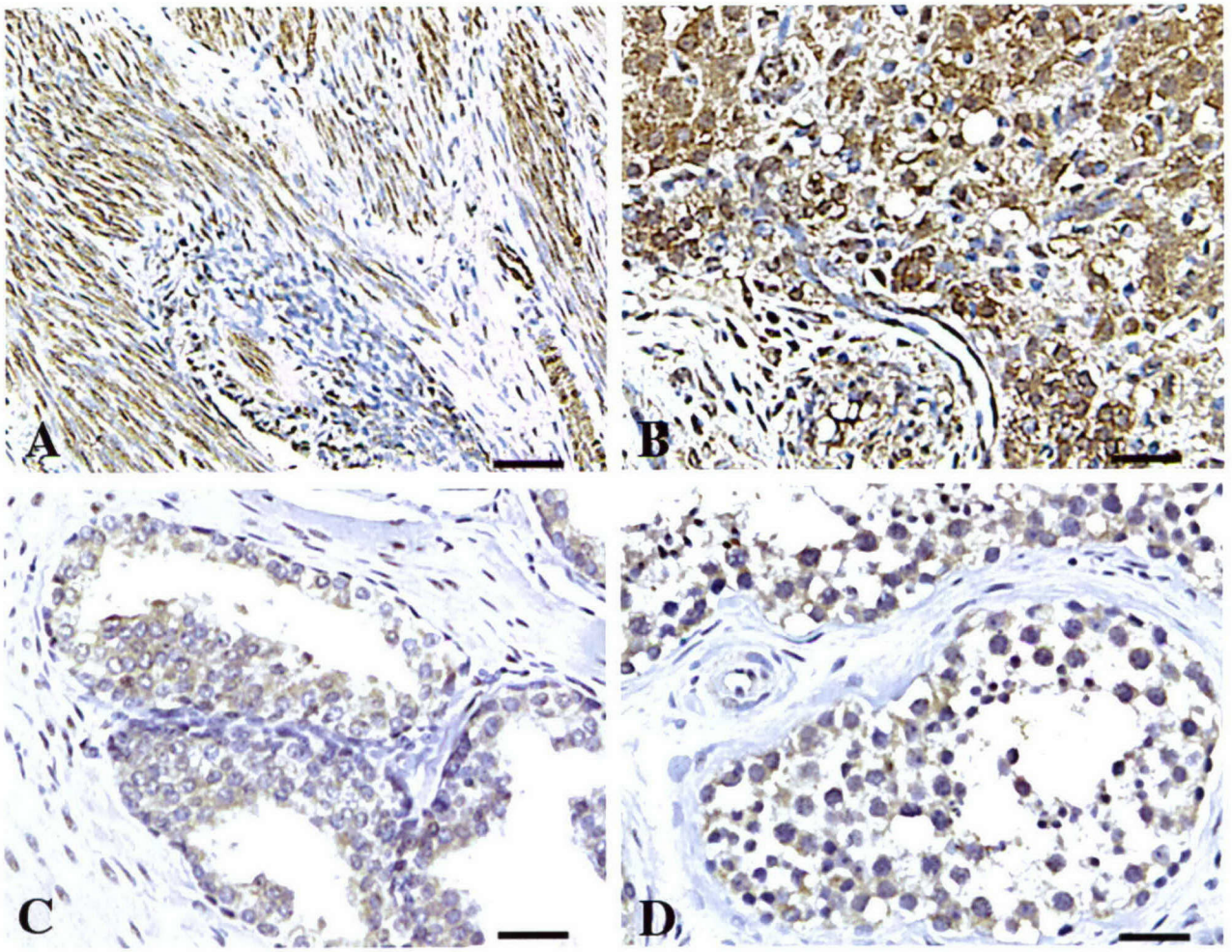


Figure 5

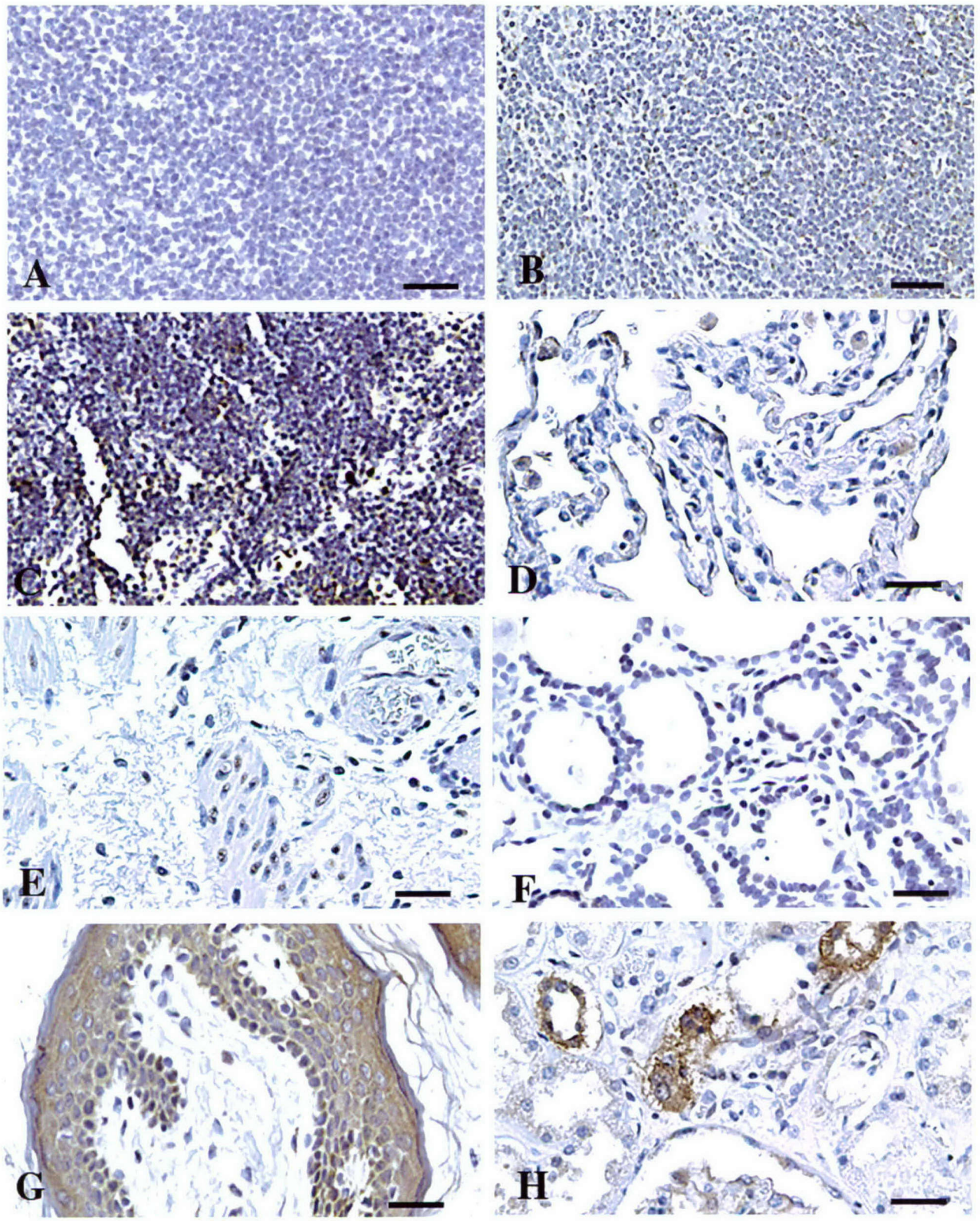


Figure 6